CaM is RyR2 bound. These results are the first quantitative in situ RyR2-CaM binding measurements.

2239-Pos Board B225
A Region Involved in Isoform-Specific Regulation of Skeletal Muscle Ryanodine Receptor by Calmodulin
Naohiro Yamaguchi, Le Xu, Gerhard Meissner.
Calmodulin (CaM) regulates both skeletal (RyR1) and cardiac (RyR2) muscle ryanodine receptor calcium channels by binding to a single highly conserved CaM binding domain (RyR1 amino acid (aa) 3614-3643; RyR2 aa 3581-3610). CaM inhibits both isoforms at an elevated Ca^{2+} concentration (micromollar), whereas at cellular resting Ca^{2+} concentration CaM has opposite effects on two isoforms (activation of RyR1 and inhibition of RyR2). This implies another region of RyR is involved in isoform-specific CaM regulation at submicromollar Ca^{2+} concentration. To identify the regions we constructed and analyzed a series of RyR1/RyR2 chimera. RyR1/RyR2 chimera carrying RyR1 aa 1-3725 is inhibited by CaM at 0.4 μM Ca^{2+} (RyR2-type), whereas chimera carrying RyR1 aa 1-4301 is activated (RyR1-type). The results suggest that RyR1 aa 3726-4301 contains a region that is responsible for CaM activation. The region overlaps with a domain resembling the sequence of CaM (CaM-like domain). Replacement of RyR1 3726-4301 with the corresponding RyR2 sequence confers CaM inhibition at $[Ca^{2+}]<1$ μM (RyR2 type). Furthermore, substitution of 5 non-conserved amino acids in RyR1 CaM-like domain with those of RyR2 (M4122T, I4123L, N4124D, F4125Y, N4130K) is sufficient for RyR2-type CaM inhibition. However, the reverse chimera and mutant RyR2 were not activated but inhibited by CaM. Taken together, the results suggest that 5 non-conserved amino acids of RyR1 are crucial for RyR1-specific CaM activation at submicromollar Ca^{2+} concentration. On the other hand, CaM inhibition of RyR2 is likely controlled by a different region. Supported by NIH (AR018687 and HL073051), NSF (EPS-0903795) and AHA (10SDG3500001).

2240-Pos Board B226
Effect of Human RyR2 CPVT Mutations on Interaction with Calmodulin
Peter Wilson, Michail Nomikos, Matthias Bochtler, F. Anthony Lai.
The ryanodine receptor (RyR) is a member of a family of intracellular calcium release channels that regulate calcium efflux from intracellular stores. The RyR2 isoform is most abundant in the heart and plays a key role in cardiac muscle excitation-contraction coupling. Clusters of mutations associated with the inherited arrhythmogenic disorder, catecholaminergic polymorphic ventricular tachycardia (CPVT), have been found in specific regions throughout RyR2, a large protein of ~5000 amino acids. Many of these CPVT mutations (total >100) are thought to occur in significant functional domains and result in the dysregulation of RyR channel function.
One such region of RyR2 is believed to comprise a calmodulin (CaM) interaction site and two EF hand motifs. The calcium-sensitive binding of CaM has been shown to regulate the opening of RyR. Hence, examining the RyR2 interaction with CaM and the potential effects of CPVT mutations on this binding may help reveal mutation-dependent mechanisms of channel dysfunction.
We have prepared bacterial expression plasmid constructs containing the wild-type human RyR2 CaM-interacting domain and introduced a series of CPVT mutations that have previously been identified to occur within this region. Expression and purification of the corresponding recombinant fusion proteins has enabled calcium-dependent binding of CaM to be determined with all these constructs. Examining the distinct functional role of calcium concentration on CaM binding kinetics and further comparative structural analyses of the wild-type and mutant RyR2 domains may help reveal the specific effect(s) that CaM-mediated regulation may have in mediating CPVT-linked arrhythmogenesis.

2241-Pos Board B227
Direct Detection of Domain Peptide Binding to the Cardiac Ryanodine Receptor (RYR2) using FRET
Florentin R. Nitu, Razvan L. Cornea, David D. Thomas, **Bradley R. Fruen**.
The RyR2 Ca release channel is activated by the synthetic domain peptide DPc10, which corresponds to a 36-residue sequence within the channel's central mutation hot-spot region. DPc10 activation is hypothesized to result from the destabilization of a critical intramolecular interaction between the N-terminal and central hot-spot regions of the full-length RyR2, and to thereby mimic the effects of arrhythmogenic mutations on this putative intramolecular interaction controlling channel activation. However, the site of DPc10 binding within the RyR2 3D structure is uncertain, and factors that may modulate binding are undefined. To directly monitor and map DPc10 binding to the RyR2, we attached a FRET acceptor at DPc10's N-terminus. A FRET donor was targeted to the RyR2 cytoplasmic assembly via a fluorescent-labeled FKBP12.6. Addition of the acceptor-labeled DPc10 (30 μM) resulted in a marked decrease in fluorescence of the RyR2-bound FKBP12.6. Fluorescence was partially restored upon FKBP12.6 dissociation from the RyR2, indicating that a major fraction of the total fluorescence decrease was attributable to FRET between FKBP12.6 and DPc10 when bound to the channel. The DPc10 dependence of FRET was similar to the DPc10 dependence of RyR2 activation observed previously in bilayer and ryanodine binding studies (EC_{50} ~25 μM), consistent with the likelihood that FRET reflected DPc10 binding at its regulatory site on the RyR2. FRET decreased as a function of increasing Ca (30 nM to 300 μM), suggesting that Ca activation of the RyR2 altered either the affinity of DPc10 binding or its proximity to the FKBP12.6 subunit. We conclude that DPc10 binds to a site on the RyR2 within 10 nm of FKBP12.6. Regulatory interactions and structural changes at this site can be monitored using FRET.

2242-Pos Board B228
β Strand Switching: A Novel Structural Rescue Mechanism in a Δexon3 Cardiac Ryanodine Receptor Mutant
Paolo A. Lobo, Lynn Kimlicka, Ching-Chieh Tung, Filip Van Petegem.
The contraction of cardiac muscle requires release of Ca2+ from the sarcoplasmic reticulum through the cardiac ryanodine receptor (RyR2). Several mutations in RyR2 are linked to inherited disorders, including triggered cardiac arrhythmias such as catecholaminergic polymorphic ventricular tachycardia (CPVT) that may lead to sudden cardiac death. A severe form of CPVT is caused by removal of an entire third exon (Δexon3) of RyR2. The 35 deleted residues form secondary structure elements which are crucial in folding of the N-terminal domain, raising the question of why the deletion is neither lethal nor confers a loss-of-function phenotype. A 2.3Å crystal structure shows that the removal results in a structural rescue: an otherwise flexible loop compensates for the loss by inserting itself into the β trefoil domain and increases the thermal stability. The other β strands in the domain show increased mobility to accommodate a sequence that bears no similarity to the deleted exon. The exon3 deletion is not tolerated in the corresponding RyR1 domain. The rescue shows a novel mechanism by which RyR2 channels can adjust their Ca2+ release properties through altering the structure of an individual domain.

2243-Pos Board B229
Crystallographic Investigation of Several Malignant Hyperthermia and CPVT Mutations in Ryanodine Receptors
Lynn Kimlicka, Filip Van Petegem.
Mutations in the Ryanodine Receptor (RYR) are known to underlie many genetic diseases. In particular, the skeletal muscle isoform (RyR1) is involved in malignant hyperthermia (MH) and central core disease (CCD), whereas mutations in the cardiac isoform (RyR2) are known to cause catecholaminergic polymorphic ventricular tachycardia (CPVT) and arrhythmogenic right ventricular dysplasia (ARVD). Despite an extensive analysis of disease mutations on the functional level, very little is known about the structural changes induced by the mutations. Here we analyze the structures and stability of over 10 different disease mutants. We present crystal structures of mutant versions of the RyR1 N-terminal disease hot spot, encoding three domains, and of the RyR2 N-terminal domain, all solved between 2.0 and 3.0 Angstrom, and compare them to the wild type structures. The observed effects on structure and stability differ substantially among the mutants. Whereas some cause a major destabilization of the overall fold, others mainly cause relative domain-domain movements or confer large conformational changes within individual domains. We discuss the likely implications of the disease mutations on the overall structure and gating properties of the intact RyR.

2244-Pos Board B230
A Model-Based Description and Burst Analysis of Purified Human Cardiac Ryanodine Receptor (hRyR2) Gating Kinetics Under Minimal Conditions
Saptarshi Mukherjee, Nia Lowri Thomas, Chloe E. Maxwell, Alan J. Williams.
Rhythmic contraction of cardiac myocytes is maintained by precisely controlled Ca^{2+} efflux from intracellular stores mediated by the cardiac ryanodine receptor (RyR2). Mutations in RyR2 can cause channel instability leading to perturbed Ca^{2+} release that can trigger arrhythmias. RyR2-dependent ventricular tachyarrhythmia is an important cause of sudden cardiac death, the mechanistic basis of which remains unclear. Most investigations of RyR2 single channel function have focussed on the secondary effects of mutations on channel function through modulation by regulatory proteins and cellular processes without emphasis on mutation-dependent effects on the gating behaviour of the channel itself. Here we describe the gating kinetics of wild-type hRyR2 when activated by its physiological trigger, cytosolic Ca^{2+} and isolated from the possible modulatory effects of regulatory factors external to the channel. Single channel experiments were performed using recombinant, purified hRyR2 under reducing conditions where the luminal Ca^{2+} was buffered at 50 nM while the cytosolic Ca^{2+} was stringently controlled using EGTA, HEDTA and NTA to achieve an activating

free Ca^{2+} range of 0-500 μM. The sigmoidal dose-response curve yields an EC_{50} of 1.45 ± 0.44 μM (n=10) and P_o saturates at ~10 μM Ca^{2+}. The gating model describes the kinetic behaviour using a minimum of three open and closed states and incorporates constitutive (unliganded) gating while also revealing very brief closings suggestive of events similar to C-type inactivation in K^+ channels. Novel detailed burst analysis of RyR2 elucidates its ligand-bound gating kinetics. The model was further validated by simulation of single channel data. This model provides a firm platform for further analysis of the effects of physiological regulatory factors and dissecting the mechanistic nature of the perturbations in mutant channels causing cardiovascular pathology. Supported by the British Heart Foundation.

2245-Pos Board B231

Trans Complementation with the Common G1885E Polymorphism Attenuates Mutant RyR2 L433P Channel Dysfunction

Aaron I. Clack, N. Lowri Thomas, Nicole C. Silvester,
Steven R. Barberini-Jammaers, F. Anthony Lai, **Christopher H. George**.

A channelopathic Leu433Pro (L433P) mutation in the cardiac ryanodine receptor (RyR2) alters intracellular Ca2+ handling and is associated with malignant arrhythmia. This mutation is reported to exclusively co-inherit with a common single nucleotide polymorphism (SNP) Gly1885Glu (G1885E) present on the same allele (i.e. in cis). We investigated the functional impact of the G1885E SNP on L433P channels. RyR2-null HEK cells were used as the background for the systematic expression of homo- and heteromeric RyR2 channels composed of recombinant wild-type (WT) subunits, and those containing L433P and G1885E substitutions. Homomeric G1885E channels were functionally normal, but Ca2+ handling abnormalities associated with the L433P mutation were exacerbated by complementation with the G1885E in cis. At the cellular level, channel dysfunction manifested as reduced caffeine-evoked Ca2+ release and decreased ER Ca2+ store content. These findings suggest that although L433P is not a typical gain-of-function mutation it augments basal channel activity. Co-expression of L433P subunits with those containing G1885E (i.e. in trans complementation) attenuated L433P mutant channel dysfunction. Cells expressing heteromeric assemblies of L433P and G1885E subunits exhibited caffeine-activation profiles, homeostatic and post-activation Ca2+ fluxes and ER Ca2+ loads that were comparable to those typical of WT channels. Our data show that the genotype, allelic distribution and the resultant mode of L433P and G1885E channel assembly (i.e. cis or trans subunit complementation) has divergent effects on RyR2 channel (dys)function. The G1885E SNP may be a complex determinant of channel behaviour in situ and the potential therapeutic benefits of normalising mutant channel dysfunction via trans-complementation strategies should be further explored.

2246-Pos Board B232

Differently from S165F, Y141H Mutation of Junctophilin-2 affects Calcium Signaling in Skeletal Muscle

Jin Seok Woo, Keon Jin Lee, Jianjie Ma, Eun Hui Lee.

Junctophilins (JPs) play an important role in the formation of junctional membrane complexes in muscle cells by physically linking the transverse-tubule and sarcoplasmic reticulum (SR) membranes. In humans with hypertrophic cardiomyopathy (HCM), mutations in JP2 are associated to altered Ca^{2+} signaling in cardiomyocytes. One of the HCM-related JP2 mutants, S165F also induces both hypertrophy and altered intracellular Ca^{2+} signaling in skeletal myotubes. Here, we tried to identify the dominant negative role of another HCM-related JP2 mutation, Y141H, in primary mouse skeletal myotubes in order to examine the effects of Y141H on skeletal muscle function. Consistent with S165F-expressing skeletal myotubes, over-expression of Y141H led to a significant increase in myotube diameter and resting cytosolic Ca^{2+} concentration. Immunoblot assays suggested that the increased resting cytosolic Ca^{2+} concentration is possibly due to the increased expression level of an extracellular Ca^{2+}-entry channel, Orai1. Unlike S165F, single myotube Ca^{2+} imaging experiments with Y141H-expressing myotubes showed a reduction in the gain of excitation-contraction coupling without an alteration in both ryanodine receptor1-mediated Ca^{2+} release from the SR and the amount of SR Ca^{2+}. Therefore, the hypertrophy in the Y141H-expressing skeletal myotubes would be differently progressed from that in the S165F-expressing skeletal myotubes.

2247-Pos Board B233

Orai1 and STIM1 Expression during Mouse Skeletal Muscle Cell Differentiation

Keon Jin Lee, Jin Seok Woo, Eun Hui Lee.

In skeletal msucle, stromal interaction molecule 1 (STIM1) is the calcium sensor of sarcoplasmic reticulum and Orai1 is an extracellular Ca^{2+}-entry channel. These two proteins are known to be components of store-operated Ca^{2+}-entry (SOCE) in skeletal muscle. Here, we examined expression of STIM1 and Orai1 during skeletal muscle cell differentiation. During differentiation of primary mouse skeletal myoblasts to myotubes, Orai1 expression was gradually increased until differentiation day 2 and kept at a certain level on further days of differentiation. STIM1 expression was increased during differentiation. Immunocytochemistry of myoblasts and myotubes with anti-STIM1 and anti-Orai1 antibodies showed that STIM1 and Orai1 were colocalized all over the cells except for nucleus parts during differentiation. These results suggest that SOCE by STIM1 and Orai1 plays a certain role in skeletal muscle differentiation.

2248-Pos Board B234

Galectin-3 Secreted by Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells Reduces Amyloid-Beta42 Neurotoxicity In Vitro

Ju-Yeon Kim, Dong Hyun Kim, Dal-Soo Kim, Ji Hyun Kim,
Sang Young Jeong, Hong Bae Jeon, Eun Hui Lee, Yoon Sun Yang, Wonil Oh, Jong Wook Chang.

In this study, we found that expression and secretion of galectin-3 (GAL-3) were upregulated by amyloid-β42 (Aβ42) exposure in human umbilical cord blood-derived mesenchymal stem cell (hUCB-MSC) without cell death. Aβ42-exposed rat primary cortical neuronal cells co-treated with recombinant GAL-3 were protected from neuronal death in a dose-dependent manner. hUCB-MSCs were cocultured with Aβ42-exposed rat primary neuronal cells or the neuroblastoma cell line, SH-SY5Y in a Transwell chamber. Coculture of hUCB-MSCs reduced cell death of Aβ42-exposed neurons and SH-SY5Y cells. This neuroprotective effect of hUCB-MSCs was reduced significantly by GAL-3 siRNA. These data suggested that hUCB-MSC-derived GAL-3 is a survival factor against Aβ42 neurotoxicity.

2249-Pos Board B235

Rapamycin is a Deceptive Tool for Probing Functional Interactions Between FK506-Binding Proteins and Ryanodine Receptors

Elisa Venturi, Samantha J. Pitt, Mano Sitsapesan, Elena Galfré,
Rebecca Sitsapesan.

Although dissociation of FKBP12/FKBP12.6 from RyR channels is widely accepted to increase open probability (Po) and induce sub-conductance gating, these effects are not observed by all investigators. Since rapamycin is frequently used to dissociate FKBP12/FKBP12.6 from RyR channels, we examined whether rapamycin itself could affect RyR function. When rabbit skeletal SR vesicles were pre-treated with 20 μM rapamycin, the Po of RyR1, in the presence of 10 μM cytosolic Ca^{2+}, was significantly higher than the Po of channels that had not been exposed to rapamycin (0.150 ± 0.055 vs 0.016 ± 0.005; SEM; n=7; $p<0.05$). Cytosolic addition of 1 μM FKBP12 did not reverse the effects of rapamycin (0.142 ± 0.043; SEM; n=3). Similarly, the Po of RyR2 significantly increased after cytosolic addition of 20 μM rapamycin in the presence of 10 μM cytosolic Ca^{2+}, from 0.039 ± 0.009 to 0.564 ± 0.072 (SEM; n=7; $p<0.0001$). Again, the effect was not reversed after perfusing away the rapamycin and after subsequent addition of 200 nM FKBP12.6. Although FKBP12 and FKBP12.6 were not able to lower the Po of rapamycin-treated channels, lowering free [Ca^{2+}] to <1 nM completely shut all channels demonstrating that sensitivity to Ca^{2+} was retained. We observed that rapamycin treatment slightly increased the frequency of resolvable sub-conductance gating states (events>3 ms duration) but, for both RyR1 and RyR2, this appeared to be correlated with elevations in Po. Similarly, slight changes in sub-conductance state gating after additions of FKBP12/FKBP12.6 were correlated with Po changes. Our data shows that dissociation of FKBP12/FKBP12.6 from RyR1/RyR2 does not influence the incidence of sub-conductance state gating. Moreover, we suggest that the direct actions of rapamycin on RyR channel gating have led to erroneous conclusions regarding the effects of FKBP12/FKBP12.6 on RyR channel function.

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2250-Pos Board B236

Effect of Phosphorylation, Cytoskeleton and FK-506 Binding Protein on the Gating of Coupled Skeletal Ryanodine Receptors

Jake T. Neumann, Sidney Fleischer, Julio A. Copello.

In skeletal muscle fibers, local Ca^{2+} sparks and global Ca^{2+} transients arise from the synchronous activation of arrays of skeletal ryanodine receptors (RyR1) in the sarcoplasmic reticulum. Marx et al. (1998) first described that synchronous Ca^{2+} signaling in cells could be explained by the coordinated gating of neighboring RyR1; i.e. coupled gating. We have previously reported that coupled gating of multiple RyR1 requires luminal Ca^{2+} as current carrier and ATP/Mg^{2+} in the cytosolic solution. As ATP is the most effective nucleotide for coupled gating in the presence of cytosolic Mg^{2+}, the role of ATP to modulate RyR1 as a substrate of protein kinases to phosphorylate the channel or to stabilize RyR1-RyR1 interactions via the cytoskeleton remains possible.